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1 **Characterisation of a small erythromycin resistance plasmid pLFE1 from the**
2 **food-isolate *Lactobacillus plantarum* M345**

3
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10 **Keywords:** erythromycin resistance, plasmid, host-range, mobilisation, *Lactobacillus plantarum*

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1 **Abstract**

2 This paper reports the complete 4031 bp nucleotide sequence of the small erythromycin resistance
3 plasmid pLFE1 isolated from the raw-milk cheese isolate *Lactobacillus plantarum* M345. Analysis
4 of the sequence revealed the coding regions for the erythromycin resistance determinant Erm(B). A
5 replication initiation protein RepB was identified belonging to the RepB proteins of the pMV158
6 family of rolling-circle replicating plasmids. The transcriptional repressor protein CopG and a small
7 counter transcribed RNA, two elements typically involved in replication control within this family
8 were also found. A putative replication initiation site including a single-strand origin (*sso*) -like
9 region succeeded by a characteristic pMV158 family double-strand origin (*dso*) was located
10 upstream of the replication region. An open reading frame following a typical origin of transfer
11 (*oriT*) site and coding for a putative truncated mobilisation (Mob) protein with a size of 83 aa was
12 detected. The product of the putative *mob* gene showed large similarity to the N-terminal region of
13 the pMV158 family of Pre/Mob proteins, but was much smaller than other proteins of this family.
14 We therefore suggest that the Mob function in pLFE1 is supplied in *trans* from another plasmid
15 present in *L. plantarum* M345.

16 Filter mating experiments showed that pLFE1 has a broad host-range with transconjugants obtained
17 from *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Listeria innocua*, the opportunistic pathogen
18 *Enterococcus faecalis* and the pathogen *Listeria monocytogenes*.

19

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21 **Keywords:** plasmid, sequence, *Lactobacillus plantarum*, erythromycin resistance gene, mobilisation

1. Introduction

Lactobacillus plantarum is a species of substantial industrial importance and has been used for centuries as starter culture in the manufacture of a broad selection of food products both for human and animal consumption. In addition, many strains of *L. plantarum* are recognized to have a general beneficial health effect, and are thus used as probiotics. *L. plantarum* is often well adapted to survive passage through the upper gastrointestinal tract and has frequently been shown to be one of the dominating *Lactobacillus* species in the human small intestine (Ahrne et al., 1998; Johansson et al., 1993).

Natural resident plasmids are common in *L. plantarum* and several papers have reported the nucleotide sequence of these: pM4 (Yin et al., 2008), pWCFS101, pWCFS102, pWCFS103 (van Kranenburg et al., 2005), p256 (Sorvig et al., 2005), pPB1 (de las Rivas et al., 2004), pMD5057 (Danielsen, 2002), pPLA4 (van Reenen et al., 1998), pA1 (Vujcic and Topisirovic, 1993), pLB4 (Bates and Gilbert, 1989) and pC30il (Skaugen, 1989). However, most plasmids in lactobacilli are small rolling-circle replicating (RCR) cryptic plasmids without any assigned function besides their own replication apparatus and occasionally mobilisation genes (Pouwels and Leer, 1993). Some documents exist on the occurrence of antibiotic resistance genes harboured on *Lactobacillus* plasmids, however, the number is relatively limited (Axelsson et al., 1988; Danielsen, 2002; Fons et al., 1997; Gevers et al., 2003; Lin et al., 1996; Tannock et al., 1994; Vescovo et al., 1982). Due to the wide environmental distribution of *L. plantarum*, the presence of antibiotic resistance plasmids in this species can be a potential health hazard. If mobile, the plasmids may be transferred to other bacteria within the food chain and in worst-case reach potentially pathogenic bacteria invading the consumer. Transfer of different *L. plantarum* plasmids harbouring resistance genes towards erythromycin and tetracycline has been documented *in vitro* to *Enterococcus faecalis* and

1 *Lactococcus lactis* (Gevers et al., 2003) and *in vivo* to *E. faecalis* using gnotobiotic rats as a model
2 of the human intestine (Jacobsen et al., 2007).
3 The present paper reports on the small *L. plantarum* erythromycin resistance plasmid pLFE1,
4 previously shown to transfer at high frequency to *E. faecalis* in the gnotobiotic rat model, but not in
5 the more complex streptomycin-treated mice model (Feld et al., 2008). The whole nucleotide
6 sequence of pLFE1 was determined and analysis was carried out in order to learn more about the
7 mechanistic basis for mobilisation, potential host-range, incompatibility grouping and expression of
8 antibiotic resistance.
9

2. Materials and methods

2.1 Bacterial strains, plasmids and culture conditions

The raw-milk cheese isolate *L. plantarum* M345 (Feld et al., 2008) previously shown to harbour the erythromycin resistance plasmid pLFE1 was used for plasmid preparation of pLFE1 and as donor in *in vitro* conjugation assays. Strain M345 was grown anaerobic at 37 °C for 24-48 h in de Man, Rogosa and Sharpe (MRS) medium (Oxoid, Hampshire, UK) supplemented with erythromycin when appropriate (for concentrations, see below).

Bacterial strains used as recipients in the conjugation study are listed in Table 1. *E. faecalis* was grown on Brain Heart Infusion (BHI) medium (Oxoid), *L. lactis* on GM17 media (M17 (Oxoid) + 10% glucose), *Bacillus* on Bacillus Cereus Selective Agar (Oxoid) and *Listeria* on Palcam (Oxoid). When appropriate, antibiotics were added to the media at the following final concentrations: 100 µg ml⁻¹ or 500 µg ml⁻¹ streptomycin (str) for selection of *Bacillus* sp. and *E. faecalis*, respectively, 500 µg ml⁻¹ spectinomycin (spec), 25 µg ml⁻¹ tetracycline (tet), 100 µg ml⁻¹ rifampicin (rif) and 100 µg ml⁻¹ nalidixic acid (nal). Erythromycin was applied at 16 or 50 µg ml⁻¹ for selection of transconjugants and donors, respectively.

2.2 Sequencing of pLFE1 plasmid

The *erm(B)* gene harboured on plasmid pLFE1 from *L. plantarum* M345 was analysed by sequencing standard PCR products. Plasmids were extracted from the strain as earlier described (Jacobsen et al., 2007) and subsequently used as template in PCR amplification of the *erm(B)* gene, as previously described (Jacobsen et al., 2007). Eight PCR reactions were set up, and the eight products pooled and purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The size of the PCR product (424 bp) was confirmed by gel electrophoresis and the sample subsequently sequenced using the *ermB*-F and *ermB*-R primers (Jensen et al., 1999). The remaining

part of the *L. plantarum* M345 *erm*(B) plasmid was sequenced by primer walking using the plasmid preparation of strain M345 as template. Initially, the primers Lor1 (5'-AGA TGA CTG TCT AAT TCA ATA G-3') and Lor3 (5'-TGC ACA CTC AAG TCT CGA-3') targeting the *erm*(B) gene and reading outwards, were applied. All sequencing reactions were performed at GATC Biotech (Konstanz, Germany).

2.3 Verification of pLFE1 structure

A restriction map of pLFE1 was made by digestion with the restriction enzymes *Xcm*I, *Xmn*I, *Ase*I, *Hinc*II, *Bss*SI and *Nde*I. As template, plasmid extraction from a pLFE1-carrying transconjugant isolate previously obtained by conjugation between *L. plantarum* M345 and *E. faecalis* JH2SS was used (Feld et al., 2008). Analysis of the plasmid profile showed that this isolate contained the pLFE1 plasmid solely. Restriction analysis was performed using restriction enzymes from New England Biolab (Herts, UK) according to the description of the manufacturer. Digested DNA was run by gel electrophoresis on a 1% agarose gel and subsequently stained with ethidium bromide. The structure of pLFE1 was further confirmed by PCR using primers *mob*₁F (5'- TGG GTC AAT CGA GAA TAT C-3') binding at position 1213-1231, *mob*₂F (5'-GAT TTG GTC AAT CGG ACA G-3') binding at position 3444-3462 and *mob*R (5'-GAA CGC AAA TAT GAG CTT C-3') binding at position 3729-3711 and 1623-1605. PCR mixtures were prepared using 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead (Amersham Biosciences, Buckinghamshire, UK) and 20 pmol of each primer in a total volume of 25 µl. Amplification using the primers *mob*₁F + *mob*R or *mob*₂F + *mob*R should result in a 286 bp and a 411 bp PCR product, respectively. PCR was carried out by initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 2 min. A final extension step at 72 °C for 10 min ended the programme.

2.4 *In vitro* mating experiment

The ability of plasmid pLFE1 to be transferred to and replicate in different Gram-positive species was assessed using a filter-mating assay. The donor and recipient strains were grown in broth supplemented with the respective antibiotics to an $OD_{600} = 0.7-1.0$. The bacteria were mixed in equal volumes and vacuum filtered onto sterile filters (HAWP04700, Millipore, Bedford, MA). The filters were placed on agar mating plates and incubated overnight at conditions optimal for the recipients. For *L. lactis* mating, GM17 media was used, for the rest BHI medium was used as mating media. All matings were performed at 37°C under aerobic conditions, except the matings with *L. lactis* and *Bacillus* as recipients, where the matings were performed at 30°C. After overnight incubation, the bacteria were washed off the filters and suitable dilutions prepared on plates selective for donor, recipients and transconjugants. Transconjugants were selected at conditions identical to their respective recipients except that erythromycin was added to the plates and the plates were incubated for 36h. Controls of separate donor and recipient plates were also prepared. The experiment was performed in triplicates.

2.5 *Exclusion of transfer via transformation and transduction*

Transformation was excluded by adding DNase I to the mating plates at a concentration of 100 µg/ml, and performing a filter mating experiment between *L. plantarum* M345 and *E. faecalis* JH2SS as described above. No difference in transfer frequency was detected (data not shown), indicating that pLFE1 is not transferred via transformation.

Two experiments were conducted to exclude that pLFE1 is transferred via transduction. In the first one, supernatant from a *L. plantarum* M345 culture was used in a filter mating experiment with *E. faecalis* JH2SS. In the second experiment, the donor and recipient were filtered on two separate filters that were placed upside down of each other, so that there was no direct contact between the

two strains. In both cases no transconjugants were detected, excluding the possibility of transduction as a mechanism of pLFE1 transfer (data not shown).

2.6 Verification of transconjugants

In order to verify that colonies isolated from selective agar plates were true transconjugants and not mutants of donor or recipient, specific PCR and fingerprinting assays were performed. The presence of the *erm*(B) gene was verified by PCR using primers specific for *erm*(B) as earlier described (Jacobsen et al., 2007). Donor and recipients were included as positive and negative controls, respectively. Random amplified polymorphic DNA (RAPD) technique was used to show similar fingerprints of recipients and transconjugants and differentiate them from the fingerprint of the donor. Template DNA of donor, recipients and transconjugants was prepared as previously described (Jacobsen et al., 2007). The PCR reaction mixtures contained 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead and 20 pmol of primer OPA-02 (Operon Biotechnologies, Cologne, Germany) in a total volume of 25 µl. The PCR programme was as follows; initial denaturation 94 °C for 4 min followed by 40 cycles of 94 °C for 1 min, 32 °C for 1 min and 72 °C for 2 min. The program was ended by extension at 72 °C for 5 min. Isolates of *Listeria monocytogenes* transconjugants were further tested by PCR using primers IntA_C_up (5'-TAG AAG TAG TGT AAA GAG CTA GAT G-3') and IntA_C_down (5'-ATA TAA AGC TTG CGG CCG CTT CTG CAA AAG CAT CAT CTG GAA AA-3') amplifying a 260 bp sequence of the virulence gene *IntA* coding for Internalin A. PCR reaction mixtures contained 5 µl DNA template, 1 PuReTaq Ready-To-Go PCR bead and 20 pmol of each primer IntA_C_up and IntA_C_down in a total volume of 25 µl. The PCR programme was as follows; initial denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and finally extension at 72 °C for 3 min. The donor and recipient were included as negative and positive controls, respectively.

All PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Bio-Rad, Waltham, MA, USA). The PCR products were run on a 1.5% agarose gel for 1h and visualized by ethidium bromide staining.

2.7 Stability of *pLFE1* in donor and transconjugants

The donor strain *L. plantarum* M345 and the obtained transconjugants from mating with *E. faecalis*, *B. subtilis*, *L. lactis*, *L. monocytogenes* and *L. innocua* were inoculated from selective (containing erythromycin) agar plates to selective liquid media and grown until exponential phase was reached (OD_{600} in the range of 0.8-1.2). The cultures were diluted 100 times by transferring 200 μ l of the culture into 20 ml non-selective media appropriate for the strain (Table 1) and incubated until stationary phase was reached. The cycle of growth and dilution in non-selective broth was repeated 8 times, which yielded approximately 53 generations according to the following equation: $g = \ln F / \ln 2$, where g is the number of generation in each cycle, and F is the dilution factor.

2.8 Erythromycin-clindamycin double-disk test

Overnight cultures of *L. plantarum* M345 were diluted 100 times, and 100 μ l of the dilution was spread on MRS agar plates. An erythromycin disk (15 μ g/ml, Oxoid) was placed 5-10 mm from a clindamycin disk (10 μ g/ml, Oxoid). The plates were incubated overnight under anaerobic conditions at 37 °C. Blunting of the clindamycin proximal to the erythromycin disk was classified as D-zone test–positive (inducible resistance), whereas no blunting of the zone was classified as D-zone test–negative (no induction).

Two *Streptococcus thermophilus* strains E2 and E18 (Tosi et al., 2007) were used as controls for inducible and constitutive resistance, respectively.

1

2 2.9 *Nucleotide sequence analysis*

3 Analysis of the complete nucleotide sequence for open reading frames (ORF) was performed using
4 the web-based NCBI ORF Finder programme. Database searches and comparisons of DNA
5 sequences or DNA-derived protein sequences were carried out using BLASTN, BLASTP and
6 BLASTX programmes (Altschul et al., 1990). Multiple sequence alignments were made with the
7 ClustalW2 program (Larkin et al., 2007) and alignments of conserved domains of proteins encoded
8 on pLFE1 was retrieved from the Conserved Domain Database (CDD) (Marchler-Bauer et al.,
9 2007). Secondary structure and calculation of free energy for hairpin formations was determined
10 using the Mfold programme (Zuker, 2003).

11

12 2.10 *Nucleotide sequence accession number*

13 The complete nucleotide sequence of pLFE1 has been deposited in the GenBank database under the
14 Accession No. **FJ374272**.

15

16

3. Results and Discussion

3.1 General features of pLFE1 sequence

The complete nucleotide sequence of plasmid pLFE1 was determined to be 4031 bp in length. The correctness of the deduced pLFE1 nucleotide sequence was verified with single digests using the restriction enzymes shown in Fig. 1 and gel electrophoresis, which confirmed the predicted nucleotide band sizes (data not shown). The average G + C content of pLFE1 is 34.4%, which is lower than that of the *L. plantarum* chromosome average of 44.5% (Kleerebezem et al., 2003), but within the range described for other small *L. plantarum* plasmids such as pA1 (34.9%) (Vujcic and Topisirovic, 1993), pPB1 (37.4%) (de las Rivas et al., 2004), p256 (37%), pWCFS101 (39.5%) and pWCFS102 (34.3%) (van Kranenburg et al., 2005). Analysis of the whole nucleotide sequence revealed 16 putative ORFs of at least 33 aa in length and one ORF smaller than 33 aa (Table 2). In a region of 3-12 nucleotides upstream of each of the ORFs, analysis was carried out for detection of ribosome-binding sites (RBS) using the *L. plantarum* RBS sequence GGAGG as consensus (Pouwels and Leer, 1993). However, in many cases either no or only poor potential RBS sites could be detected and translational coupling to other putative proteins did not seem plausible, and these ORFs were considered artefacts.

3.2 Replication initiator (RepB) protein

A putative replication initiator (RepB) protein was identified at position 2421-3059 (212 aa) (Fig. 1 + Fig. 2), showing high similarity to the RepB proteins of the *L. plantarum* rolling-circle replicating (RCR) plasmid pLB4 (80% identity over 211 aa) (Bates and Gilbert, 1989), RCR plasmid pLF1311 from *Lactobacillus fermentum* (82% identity over 206 aa) (Aleshin et al., 1999) and RCR plasmid pBM02 (71% identity over 205 aa) from *L. lactis* subsp. *cremoris* (Sanchez and Mayo, 2003). The putative RepB protein showed a conserved domain architecture corresponding to that of Rep_2

plasmid replication proteins (pfam01719) in the Conserved Domain Database (CDD). The sequence included five motifs (Fig. 2 + Fig. 3) typical for plasmid family pMV158 (del Solar et al., 1993). These motifs include the putative binding site for Mg^{++} (motif R-III), which is thought to be required for nicking activity and the active tyrosine residue (motif R-IV and/or motif R-V) involved in the catalytic attack of the plasmid DNA during initiation of replication (del Solar et al., 1993; del Solar et al., 1998; Dyda and Hickman, 2003; Ilyina and Koonin, 1992).

3.3 Plasmid copy number control

A putative gene coding for CopG (59 aa) previously referred to as RepA was found at position 2118-2297 bp (Fig. 1 + Fig. 2), showing high similarity to RepA (70% identity over 51 aa) of pLB4 (Bates and Gilbert, 1989), RepA (70% identity over 51 aa) of pLF1311 (Aleshin et al., 1999), RepA (68% identity over 51 aa) of the *L. lactis* plasmid pAR141 (Raha et al., 2006) and RepA (69% identity over 46 aa) of pBM02 (Sanchez and Mayo, 2003). The putative CopG protein revealed a conserved sequence structure similar to the RHH_1 ribbon-helix-helix family of CopG repressor proteins (pfam01402 in the Conserved Domain Database) (Fig. 4).

CopG is a transcriptional repressor protein that regulates the plasmid copy number in the pMV158 derivative plasmid pLS1 (del Solar et al., 1995; del Solar and Espinosa, 1992). By binding to the single *copG-repB* promoter region, CopG prevents host RNA polymerase binding and thus represses the synthesis of the replication initiation protein RepB as well as itself (del Solar et al., 1990). A structure of the replication region comparable to pLS1 has also been revealed in other plasmids belonging to the pMV158 family suggesting that they are controlled in a similar fashion (Bates and Gilbert, 1989; Cocconcelli et al., 1996; Raha et al., 2006; Vujcic and Topisirovic, 1993). Sequence analysis of pLFE1 indicated a homologous architecture with a putative promoter 18 nucleotides upstream of the *copG* start codon with a -35 (TTGTAT) and -10 (TATAAT) sequence

1 in a distance of 17 base pairs and typical RBS sites located upstream each gene, *copG* and *repB*
2 (Fig. 2). However, no sequence matching the criteria of a 13-bp pseudo symmetric element
3 overlapping the -35 *copG-repB* promoter box could be found. This element was demonstrated to be
4 the target binding site of CopG in pLS1 (del Solar et al., 1990) and similar sequences have been
5 detected in other pMV158 family plasmids, although their function have not been elucidated (del
6 Solar et al., 2002).

7 The active DNA binding unit of CopG from plasmid pMV158 has been shown to display a
8 characteristic ribbon-helix-helix (RHH) motif, which is built up around a glycine residue mediating
9 a turn between two α -helices. At conserved positions of the α -helices specific residues are required
10 to maintain the hydrophobic core of the motif (Gomis-Ruth et al., 1998). Within the putative *copG*
11 gene of pLFE1 the presence of a glycine residue and specifically positioned hydrophobic residues
12 were established (Fig. 4).

13 Apart from CopG, a small countertranscribed RNA (ctRNA) of approximately 50 nucleotides in
14 size has been shown to control the plasmid copy number of pLS1 (del Solar et al., 1995; del Solar et
15 al., 1997). The ctRNA is transcribed in the opposite direction of the mRNA encoding the RepB
16 protein and thus inhibits translation of RepB by binding to a region of the *copG-repB* mRNA,
17 which includes the RBS of *repB* (del Solar et al., 1997). Similar genetic organizations of ctRNA are
18 present in other members of the pMV158 family. Thus, the coordinate action of the two plasmid-
19 encoded elements CopG and ctRNA has therefore been suggested to be a common mode of plasmid
20 copy number regulation in the pMV158 plasmid family (del Solar and Espinosa, 1992). Indeed,
21 sequence analysis of pLFE1 revealed a putative ctRNA encoded on the complementary strand in the
22 region between *copG* and *repB* (Fig. 1 + Fig. 2). Within the amino-terminal region of *repB* a
23 putative promoter consisting of less conserved -10 (TTTCAT) and -35 (TAGGCA) boxes in a
24 distance of 18 nucleotides was detected. A possible transcriptional terminator was found

overlapping the carboxyl-terminal end of *copG*. This plausible terminator could be a *rho*-independent site, containing an inverted repeat configuring a potential hairpin structure followed by a T-stretch (depicted by an A-stretch at the complementary strand in Fig. 2).

3.4 Origin of replication

In RCR plasmids the double-strand origin (*dso*) is the initiation site of leading-strand synthesis. The *dso* contains a *nic* locus, which generally is well conserved within each plasmid family and a binding site (the *bind* locus) showing larger sequence variation (Khan, 1997). Within pLFE1 a region of 22 bp (5'-atGGGGGcACTACGACaCCCCC-3') showing high similarity (four mismatches) to the pMV158 family *nic* loci (del Solar et al., 1998) was found 162 bp upstream of *copG* at position 1934-1955 bp (Fig. 1 + Fig. 2). This region showed 100% similarity to the *dso* of both *L. fermentum* (Aleshin et al., 1999) and the shuttle vector pLF14 (unpublished, Accession No. **X85436.1**). The putative nick sequence and the corresponding nick site (5'-cACTACG/AC-3') corresponds well (one mismatch) with the previously defined consensus sequence for the pMV158 family (Moscoso et al., 1995). The nick sequence of pLFE1 is contained within an inverted repeat (IR5 with a calculated free energy $dG = -8.4$ kcal/mol) and flanked by a three direct repeats identical to those composing the Proximal Direct Repeats (PDR) of the *nic* locus from plasmid pE194 (Ruiz-Maso et al., 2007) (Fig. 2). Direct repeats constituting the Distal Direct Repeats (DRR) or *bind* locus identified in several RCR plasmids, but not all, of the pMV158 family (Ruiz-Maso et al., 2007) could not be identified on pLFE1. In other RCR plasmids, binding and interaction with RepB has been shown to change the characterised secondary structure resulting in creation of a hairpin, which in turn exposes the nick sequence and thus initiates replication (Khan, 1997).

1 A region of 266 nucleotides immediately upstream of the putative *dso* showed a large potential of
 2 palindromic structures (dG = -50.6 kcal/mol) (Fig. 1 + Fig. 2). We propose that this region
 3 constitutes the single-strand origin (*sso*) of pLFE1 from where lagging-strand synthesis is initiated.
 4 *Ssos* are by substantial secondary structure, but the sequence similarity may be very low even
 5 between plasmids belonging to the same family (Gruss and Ehrlich, 1989; Khan, 1997).
 6 Comparison of the putative *sso* sequence of pLFE1 with four types of *ssos* (*ssoA*, *ssoU*, *ssoT* and
 7 *ssoW*) previously described (Kramer et al., 1999) showed no general sequence similarity (data not
 8 shown). However, an RS_B (recombination site) or RS_B-like site (5'-TTTTTCGTCGGCATAA-3')
 9 was recognized at position 1669-1683 (Fig. 2) at the basis of the stem of a putative hairpin structure
 10 (dG for IR1 = -33 kcal/mol). This site has apart from being a recombination site been proposed to
 11 be involved with host RNA polymerase binding to the *sso*. The RS_B may therefore be critical for the
 12 synthesis of a primer RNA that can be used to initiate lagging-strand replication. The RS_B was first
 13 recognized in *ssoA* origins, but homologues have been detected in the other types of *ssos* as well
 14 (Kramer et al., 1999).
 15 A second region of plasmid pLFE1 potentially containing an origin of replication was found in the
 16 non-coding area downstream the putative *mob* gene. A sequence of 20 bp (5'-
 17 TtcTTCTTATCTTGATAcTA-3'), which showed high similarity (three mismatches) with pC194
 18 family *dsos* was found at position 4009-4028 (Fig. 1) (del Solar et al., 1998; Wu et al., 2007). This
 19 sequence showed 100% identity with the putative *dso* of the *L. plantarum* plasmids pLP1 (Bouia et
 20 al., 1989) and pC30il (Skaugen, 1989) and the *L. lactis* plasmid pWC1 (Pillidge et al., 1996).
 21 Upstream of the putative *dso* another potential *sso* region of 240 nucleotides (Fig. 1), which showed
 22 high potential of palindromic structures (dG = -63.2 kcal/mol) was detected. This region contained
 23 an RS_B-like sequence 100% identical to the one described above, which also was placed at the basis
 24 of the stem of a potential hairpin structure (dG = -35.2 kcal/mol). The sequences pursuing these two

identical RS_B-like sequences were 75% identical with the similarity discontinuing upstream of the putative *dsos* (data not shown). Thus, despite the lack of overall sequence similarity to any previously known *ssos*, we suggest the presence of two *ssos* within pLFE1. The existence of two functionally intact *ssos* simultaneously present on a single plasmid has earlier been reported for pMV158, which has been shown to contain both an *ssoA* (del Solar et al., 1987) and *ssoU* type (van der Lelie et al., 1989).

3.5 Erythromycin resistance

A putative antibiotic resistance gene *erm*(B) conferring resistance to macrolide, lincosamides and streptogramin (MLS) antibiotics was located at position 625-1362. The *erm*(B) gene encodes a protein 100% identical to the erythromycin ribosome methylase Erm(B) of *Enterococcus faecium* (De Leener et al., 2005). The group of Erm(B) proteins is well-conserved showing 98-100% similarity at the nucleotide level (Roberts et al., 1999). Erm(B) enzymes confer resistance by adding one or more methyl groups to a specific adenine residue located in the 23S rRNA subunit thereby preventing binding of the antibiotic (Weisblum, 1995a).

A small region (27 aa) was identified 124 nucleotides upstream the *erm*(B) start codon, which at its full length showed 100% identity with MLS leader peptides from a range of mobile elements harboured in different bacterial species. The functional role of MLS leader peptides is to regulate expression of the erythromycin methylase by conformational changes of the secondary structure of the mRNA, which affects the neighbouring region upstream of the methylase (Weisblum, 1995b).

In the absence of erythromycin, translation of the leader peptide is active. Hereby, the mRNA assumes a configuration that positions the RBS site and initial codons of the methylase within the stem of a hairpin structure, thus preventing its translation. However, when erythromycin is present, it binds to and suspends ribosomes involved in translation of the leader peptide. This in turn results

1 in naked mRNA, encoding the remainder of the leader ORF, this additional free RNA gives rise to
2 another conformation of RNA stem-loops than when translation of the leader peptide occurs and
3 thereby exposes the RBS site of the methylase thereby promoting its translation (Min et al., 2008a;
4 Weisblum, 1995b).

5 Several isolates have been reported constitutively to produce erythromycin methylase although
6 putative MLS leader peptides were detected (Gfeller et al., 2003; O'Connor et al., 2007). However,
7 the putative MLS leader peptide of pLFE1 is identical to leader peptides from strains of
8 *Streptococcus pneumoniae*, which have been reported functional (Okitsu et al., 2005). Compared to
9 the leader peptide in the *erm(B)* gene of Tn917 (Shaw and Clewell, 1985) the peptide in pLFE1 is
10 only 27 aa i.e. 9 aa shorter due to a single mutation resulting in an earlier stop codon. A previous
11 study suggested that a similar mutation caused an increased level of methylase translation, resulting
12 in an apparently constitutive resistant phenotype. The mutation increased the basal methylase
13 activity by approximately 3.8 fold and the induced activity by approximately 4.9 fold (Oh et al.,
14 1998). However, there are also reports where this shortening in the length of the leader peptide has
15 no influence on the expression of *erm(B)*, i.e. the leader peptide is still functional (Min et al.,
16 2008b; Min et al., 2008a). Using the erythromycin-clindamycin double-disk test, the strain *L.*
17 *plantarum* M345 was assigned to the constitutive resistance phenotype (data not shown). This
18 constitutive expression may be caused by a change in nucleotide just after RBS for *erm(B)*.
19 Compared to the sequence of the Tn917-like transposon LP-1 from *Streptococcus pneumoniae*
20 (Okitsu et al., 2005) harbouring an inducible phenotype, there is a shift from GGAGTG to
21 GGAGAG. Min and co-workers (Min et al., 2008a) showed that a shift in this sequence resulted in
22 destabilization of a stem loop resulting in an increased basal level of expression. Using the Mfold
23 programme, the RBS for *erm(B)* was fully exposed, confirming the constitutive resistance
24 phenotype.

3.6 Mobilisation region

A truncated recombinase/mobilisation (*pre/mob₁*) gene (Fig. 1) was found at position 1386-1606, which showed 100% nucleotide identity with the carboxyl-terminal end of the *Lactobacillus sakei* plasmid pYS18 *pre/mob* gene (unpublished, Accession No. **EU185047**) and 94% nucleotide identity with the *pre/mob* gene of *L. lactis* subsp. *lactis* plasmid pK214 (unpublished, Accession No. **YP_001429536**). However, despite the presence of an ATG start codon, no potential RBS or transcriptional coupling could be detected (Table 2) thus rendering translation of this protein unlikely.

In a different region of pLFE1 a second truncated *pre/mob₂* gene was found at position 3312-3563 bp. A strong RBS site (AGGAG) was located seven nucleotides upstream of the ATG start codon and a possible promoter was found with a -35 box (TTACGA) and a -10 box (TATACT) in a distance of 17 nucleotides (Fig. 5). The *pre/mob₂* gene showed 94% nucleotide identity with the amino-terminal end of the *pre/mob* gene of pK214 and 81% nucleotide identity with the *pre/mob* gene of pYS18. Sixty-one nucleotides downstream of the *pre/mob₂* stop codon a fragment showing 93% nucleotide identity with the carboxyl-terminal end of the pK214 *pre/mob* gene and 96% nucleotide identity with the *pre/mob* gene of pYS18 was found (Fig. 5). However, no start codon or putative RBS could be identified for this small fragment.

The odd sequence structure of pLFE1 with several truncated *pre/mob* genes or gene fragments was verified by PCR using primers *mob₁F*, *mob₂F* and *mobR* (data not shown). The *mob₂F* + *mobR* primers were specifically designed to confirm the short distance of 61 nucleotides and thus lack of a middle region of the *pre/mob* gene between the amino-terminal *pre/mob₂* gene and the carboxyl-terminal *pre/mob* fragment. *Mob₁F* + *mobR* were designed to confirm the spatial separation of *pre/mob₁* and *pre/mob₂* with the former placed downstream of *erm(B)*.

1 The Pre/Mob proteins of pK214 and pYS18 belong to the pMV158 family of Pre/Mob proteins that
2 functions both in mobilisation and recombination (Pre for plasmid recombination enzyme) (Francia
3 et al., 2004). The Pre/Mob family contains within the amino-terminal region three highly conserved
4 motifs considered to form part of the catalytic centre of the relaxase enzyme. In the *pre/mob₂* region
5 of pLFE1 only two of the three motifs could be identified (Fig. 5). Additionally, the size of the
6 putative Pre/Mob₂ is only 83 aa in contrast to other considerably larger Pre/Mob proteins
7 (approximately 350-500 aa) belonging to this family (de las Rivas et al., 2004; Gennaro et al., 1987;
8 Josson et al., 1990; Somkuti and Steinberg, 2007).

9 Mobilisation proteins normally demonstrate relaxase activity and are thus essential in preparation of
10 the plasmid for transfer (Francia et al., 2004). In plasmid pMV158 the Pre/Mob protein has been
11 shown to nick supercoiled plasmid DNA at the origin of transfer (*oriT*) (Guzman and Espinosa,
12 1997) and without the presence of an intact Pre/Mob protein conjugal transfer was suspended
13 (Priebe and Lacks, 1989; van der Lelie et al., 1990). Since pLFE1 has been shown transferable
14 (Feld et al., 2008) but does not seem to contain an intact Pre/Mob protein, we suggest that it must
15 be supplied in *trans* in *L. plantarum* M345. This organization has been shown functional in
16 *Bacillus*, where transfer-deficient Mob⁻ hybrid plasmid constructs of pUB110 and pBC16 could be
17 mobilised by complementation of a Mob protein in *trans* (Selinger et al., 1990). It was excluded by
18 *in vitro* transfer experiments that pLFE1 is transferred via transformation or transduction (data not
19 shown). Apart from relaxase activity, which could be provided in *trans*, the presence of an *oriT*
20 region in *cis* has been shown to be a minimal requirement for mobilisation of plasmids such as
21 pUB110 and pBC16 (Selinger et al., 1990). The *oriT* site is very similar among members of the
22 pMV158 family and is located upstream of the *mob* gene, overlapping its promoter. The *oriT* has a
23 conserved hairpin structure with an IR of 7-10 nucleotides forming the stem and a loop of usually 6
24 nucleotides presenting the nick sequence (Francia et al., 2004; Guzman and Espinosa, 1997). A

putative *oriT* site matching these criteria was identified in pLFE1 at position 3257-3276, 34 nucleotides upstream of the *pre/mob*₂ start codon. A putative -10 promoter box was embedded within this sequence and an IR consisting of 8 nucleotides interrupted by 6 nucleotides was also recognized (Fig. 5).

3.7 Plasmid recombination

Apart from being the origin of transfer, *oriT* also functions as a putative recombination site (RS_A). The plasmid-encoded Pre/Mob protein mediates site-specific recombination at RS_A in *trans* (Gennaro et al., 1987). Analysis of several plasmids has shown a marked sequence divergence at the RS_A site with the one side showing significant similarity to one plasmid and the other side showing similarity to a second plasmid – thus indicating development by recombination of the plasmids at their respective RS_A sites (Hauschild et al., 2005; van der Lelie et al., 1989). In pLFE1 a similar organization was detected where the sequence including and upstream RS_A exhibits 96% nucleotide identity with a region of the *L. lactis* plasmid pK214, yet the sequence immediately downstream RS_A presents no significant similarity to pK214 but 96% identity with regions of the *L. plantarum* plasmids pPLA4 (van Reenen et al., 1998) and pPB1 (de las Rivas et al., 2004) (data not shown). However, pLFE1 has a very unusual sequence structure amongst others with several interrupted *pre/mob* regions suggesting that pLFE is the product of several relatively recent recombination events. The presence of *pre/mob*₁ could be the result of recombination with pYS18, since a fragment of 308 nucleotides including *pre/mob*₁ shows 100% nucleotide identity with pYS18. The similarity starts with G in the putative ATG start codon and the complete identity continues ten nucleotides downstream of the RS_B recombination site (data not shown).

3.8 Host range and stability of pLFE1

A filter-mating assay was used to assess the ability of pLFE1 to be mobilised to and replicate in different Gram-positive bacteria. Potential pLFE1-carrying transconjugants were isolated on selective agar plates from matings with *E. faecalis*, *Bacillus subtilis*, *L. rhamnosus*, *L. lactis*, *Listeria monocytogenes* and *Listeria innocua* recipients (Table 3). These isolates were confirmed to be true transconjugants by PCR using specific primers, RAPD fingerprinting and plasmid analysis (data not shown). No transfer was detected to the recipient *B. thuringiensis* under the experimental conditions applied (Table 3). However, transfer and maintenance of pLFE1 in this species cannot be excluded from the present results, but may be possible under different conditions.

The stability of pLFE1 in its original host and the obtained transconjugants was tested. During growth for 53 generations without selective pressure, pLFE1 was 100% stable in all species tested, except *L. monocytogenes* where 93% of the tested cells retained the plasmid (data not shown).

The *in vitro* mating experiment included in this study established the ability of a broad range of Gram-positive species to function as hosts of pLFE1 and to stably maintain the plasmid. An important factor for the stable maintenance of RCR plasmids in a host is the presence of a compatible *sso*, since lack of this will result in accumulation of single-stranded plasmid DNA (Leer et al., 1992; van der Lelie et al., 1989) and subsequently segregation (Gruss and Ehrlich, 1989). The efficiency of the host RNA polymerase to bind to the plasmid *sso* promoter sequence has earlier been suggested as a key factor deciding the compatibility between the host and the plasmid (Kramer et al., 1998; Kramer et al., 1999). In pLFE1, the observed broad host-range could imply the presence of an *ssoU* type of *sso*, since the other types of *ssos* seem to function optimally only in their natural hosts, possibly due to a more specific binding site. In contrast, *ssoU* has been isolated from a broad range of Gram-positive bacteria such as *B. subtilis*, *Staphylococcus aureus*, *S. pneumoniae* and *L. lactis* (Kramer et al., 1999).

1 Analysis of the pLFE1 nucleotide sequence failed to detect the presence of a typical *mob* gene, but
2 rather revealed a number of truncated genes or fragments coding for proteins with similarity to the
3 pMV158 family of recombination and mobilisation proteins. Mobilisation of members of this
4 family can be facilitated by a wide range of helper plasmids such as the Gram-positive conjugative
5 plasmids pIP501, pAM β 1, pLS20, pXO11, pXO12 and pAD1 as well as Gram-negative broad host-
6 range plasmids belonging to incompatibility group IncW, IncF and IncP and conjugative
7 transposons like Tn916, Tn925 and Tn1545 (for a review, see (Francia et al., 2004). However,
8 whether the *mob* genes found in pLFE1 encode functional Mob proteins and can provide the
9 relaxase activity needed to initiate plasmid transfer is uncertain and requires further study.
10 However, we suggest that the *mob* function of pLFE1 is supplied in *trans* from another plasmid
11 present in the *L. plantarum* M345 host. If a functional *mob* gene is absent in pLFE1, it significantly
12 reduces the probability of dissemination of pLFE1 from hosts other than *L. plantarum* M345.
13 Hence, in order to sustain transfer, both a *mob* gene compatible with the pMV158 type of *oriT* and
14 several *tra* genes facilitating the mating channel between the new host and recipient will be required
15 in *trans*.
16
17

1 **4. Conclusion**

2 In this paper we have analysed the nucleotide sequence of plasmid pLFE1 and found two regions
3 containing a putative double-strand origin and single-strand origin suggesting that pLFE1 replicates
4 via a rolling-circle mechanism. Furthermore, analysis of the replication region places pLFE1 in the
5 pMV158 family of RCR plasmids. We have shown that pLFE1 has a broad-host range and is stably
6 maintained in its new hosts, however the presence of a typical *mob* gene could not be confirmed.
7 Instead, several truncated genes encoding proteins with similarity to the pMV158 family of
8 Pre/Mob proteins were detected, and analysis of the Mob function in pLFE1 requires further study.

9

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14

1 **Legends to figures**

2 Fig. 1

3 Map of plasmid pLFE1 showing elements described in the text and restriction sites for enzymes
4 used for nucleotide sequence verification. (black arrows: ORFs; grey arrow: ctRNA; grey boxes:
5 *ori T*, *ssos* and *dsos*.

6

7 Fig. 2

8 Detailed DNA sequence of pLFE1 replication region. Inverted repeats (IR) are underlined and
9 proximal direct repeats (PDR) are double underlined. The RS_B-region of the putative *ssu* is shown
10 in bold and the *dso* nick site is indicated by an arrow. The deduced amino acid sequences of CopG
11 and RepB are shown and putative –35 and –10 promoter boxes and ribosome binding sites (RBS)
12 are underlined with a slashed line. The start and stop codons are marked in bold as are the five
13 conserved motifs of repB. The putative promoter and terminator regions of the ctRNA are shaded.
14 Finally the five conserved motifs (see Fig. 3) of Rep proteins from the pMV158 family are marked
15 in bold.

16

17 Fig. 3

18 Multiple sequence alignment of RepB proteins belonging to Rep_2 plasmid replication proteins
19 (pfam01719) in the conserved domain database (CDD). Motifs typical of plasmid family pMV158
20 are shown according to (del Solar et al., 1993). Numbers to the left indicate the position of the
21 motifs in each plasmid.

22

23 Fig. 4

1 Multiple sequence alignment of CopG proteins belonging to RHH_1 ribbon-helix-helix CopG
2 repressor proteins (pfam01402) in the conserved domain database (CDD). The glycine residues
3 mediating the turn between the two helices are framed. Conserved positions with hydrophobic
4 residues are marked with grey, as are the highly conserved Thr/Ser residues in the turn between
5 helix A and helix B (after (Gomis-Ruth et al., 1998). Numbers to the left and right indicate the
6 aligned start and end position of the protein sequences.

7

8 Fig. 5

9 Sequence analysis of the putative *pre/mob₂* region of pLFE1. The deduced amino acid sequence and
10 the two conserved motifs are indicated as is the amino acid sequence corresponding to the small
11 *pre/mob* carboxyl-terminal fragment. The putative RBS is indicated in small letters and predicted –
12 10 and –35 promoter box sequences are underlined. The nucleotide sequence written in bold is the
13 potential *oriT* region, which includes the IR forming the hairpin structure and the nick
14 site, indicated in italic and with an arrow, respectively.

15

1

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9

Table 1

Strains used

| Strains | Characteristics* | Source or reference |
|--|--------------------------------------|--------------------------|
| <i>Lactobacillus plantarum</i> M345 | Plasmid pLFE1, <i>erm</i> (B) | Feld et al., 2008 |
| <i>Enterococcus faecalis</i> OG1SS | Str ^r , spec ^r | Franke and Clewell, 1981 |
| <i>E. faecalis</i> JH2SS | Str ^r , spec ^r | Tomich et al., 1980 |
| <i>Lactobacillus rhamnosus</i> VTT E-042730 | Tet ^r | VTT |
| <i>Lactococcus lactis</i> BU2-60 | Str ^r , Rif ^r | Neve et al., 1984 |
| <i>Listeria monocytogenes</i> LO28 | Nal ^r | Vicente et al., 1985 |
| <i>Listeria innocua</i> DSM 20649 | Nal ^r | DSMZ |
| <i>Bacillus subtilis</i> AW59 | Str ^r | Wilcks, A., unpublished |
| <i>Bacillus thuringiensis</i> GBJ01 | Str ^r | Jensen et al., 1995 |

* The r indicates resistance to the following antibiotics streptomycin (str), spectinomycin (spec), tetracycline (tet), rifampicin (rif), nalidixic acid (nal). DSMZ: German Collection of Microorganisms and Cell Cultures. VTT Culture Collection, Finland.

Table 2

Description of putative ORFs in pLFE1. ORFs marked in bold have a high probability of translation as predicted from RBS sites and potential translational coupling.

| Name | strand | Size | Position | RBS | % | aa | Best BLAST match | Accession no. |
|-------------------------------|--------|------|-----------|--------------|--------------|----|---|-----------------|
| | | (aa) | (bp) | | identity | | | |
| <i>Erm</i> (B) leader peptide | + | 27 | 417-500 | GGAGG | 100 (27 aa) | | MLS leader peptide, <i>Streptococcus pneumoniae</i> , transposon Tn6003 | <u>AM410044</u> |
| <i>Erm</i> (B) | + | 245 | 625-1362 | <u>GGAGA</u> | 100 (245 aa) | | ErmB, Erythromycin ribosome methylase of <i>E. faecium</i> clone 1 | <u>AAX12187</u> |
| ORF 3 | + | 34 | 1166-1270 | — | | | no significant similarity | |
| ORF 4 | + | 64 | 1307-1501 | <u>GTACC</u> | 73 (34 aa) | | hypothetical protein, <i>Streptococcus cristatus</i> | <u>AAV63933</u> |
| <i>pre/mob</i> _I | + | 73 | 1384-1605 | — | 100 (72 aa) | | Mob, mobilization protein of <i>Lactobacillus sakei</i> plasmid pYS18 | <u>ABW71679</u> |
| <i>CopG</i> | + | 59 | 2118-2297 | <u>AGAGA</u> | 70 (51 aa) | | RepA, plasmid copy number control protein of <i>L. plantarum</i> RCR plasmid pLB4 | <u>P20044</u> |

| | | | | | | |
|----------------------------|---|-----|---------------|-----------------------------|--|---------------------|
| ORF 7 | + | 42 | 2272- 2400 | CAAGC | no significant similarity | |
| <i>RepB</i> | + | 212 | 2421- 3059 | <u>GGAAG</u> 80 (211 aa) | RepB, replication initiation protein of <i>L. plantarum</i> RCR plasmid pLB4 | <u>P20045</u> |
| ORF 9 | + | 33 | 3077- 3178 | – | no significant similarity | |
| <i>pre/mob₂</i> | + | 83 | 3312- 3563 | <u>AGGAG</u> 95 (83 aa) | Mob, mobilization protein of <i>L. lactis</i> subsp. <i>lactis</i> plasmid pK214 | <u>YP_001429536</u> |
| ORF 11 | + | 33 | 3442- 3543 | – | no significant similarity | |
| ORF 12 | + | 35 | 3800- 3907 | AGCGT | no significant similarity | |
| ORF 13 | – | 59 | 338-159 | TAAGA 31 (61 aa) | C-terminal domain containing protein, <i>Tetrahymena thermophila</i> | <u>XP_001013113</u> |
| ORF 14 | – | 72 | 1560- 1342 | – | 33 (56 aa) hypothetical protein, <i>Paramecium tetraurelia</i> | <u>XP_001449055</u> |
| ORF 15 | – | 41 | 1735- | <u>GCAAG</u> | no significant similarity | |

| | | | | | | | |
|--------|---|----|---------------|--|--|------------------------|--|
| | | | 1610 | | | | |
| ORF 16 | – | 36 | 1763- 1653 | <u>G</u> <u>C</u> <u>A</u> <u>A</u> A | no significant similarity | | |
| ORF 17 | – | 65 | 3176- 2979 | <u>A</u> <u>G</u> <u>C</u> <u>G</u> <u>C</u> | 34 (58 aa) rCG54873, isoform CRA_a, <i>Rattus norvegicus</i> | <u>EDL98520</u> | |

Table 3

Mobilisation of pLFE1 from *Lactobacillus plantarum* M345 to various Gram-positive recipients

| Recipient | Transfer frequency* (Transconjugants/recipient) |
|------------------------------------|---|
| <i>E. faecalis</i> JH2SS | 9.8×10^{-9} (1.3×10^{-8}) |
| <i>E. faecalis</i> OGISS | 1.6×10^{-7} (1.0×10^{-7}) |
| <i>L. lactis</i> BU2-60 | 2.4×10^{-8} (9.2×10^{-10}) |
| <i>B. subtilis</i> AW59 | 3.4×10^{-9} (3.4×10^{-9}) |
| <i>B. thuringiensis</i> GBJ01 | $<1.0 \times 10^{-9}$ |
| <i>Listeria innocua</i> BSM 20649 | 7.1×10^{-8} (2.5×10^{-8}) |
| <i>Listeria monocytogenes</i> LO28 | 3.6×10^{-6} (2.8×10^{-6}) |

* Results are average from triplicates and standard deviations are given in brackets.

Figure 1

Figure 1

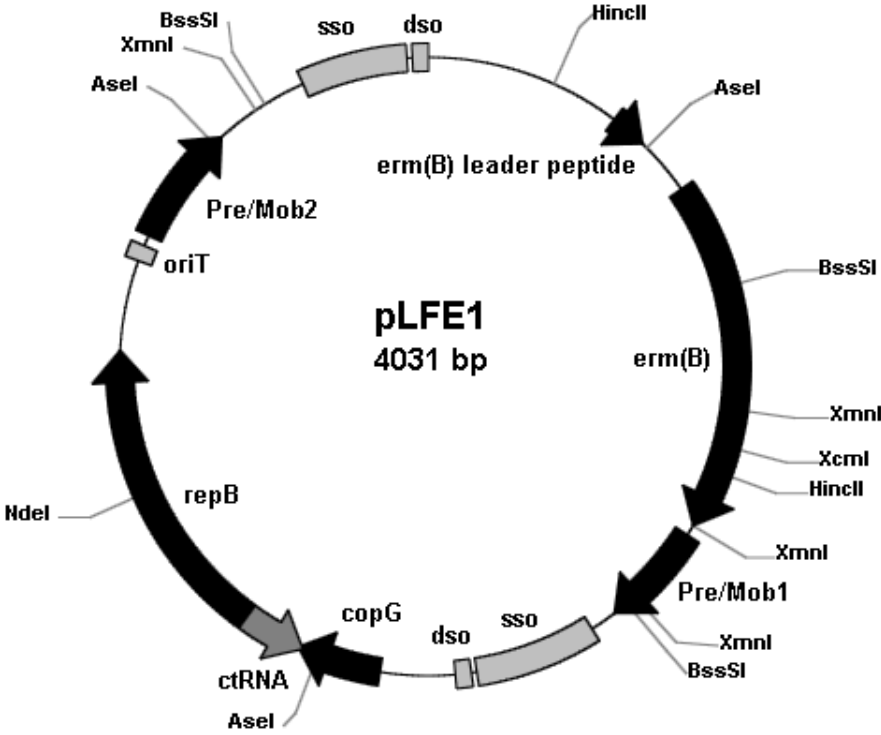
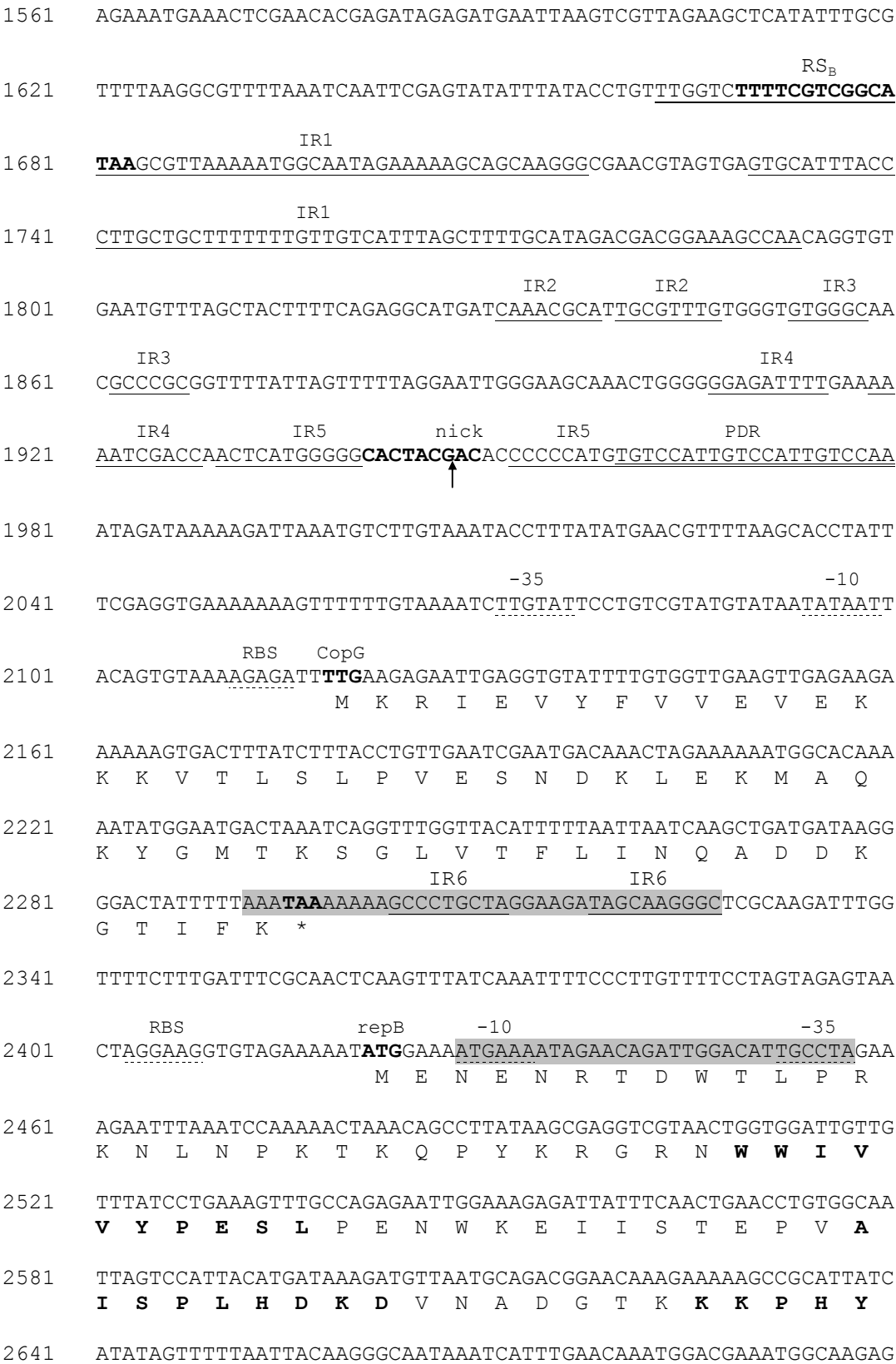


Figure 2



H I V F N Y K G N K S F E Q M D E M A R

2701 CTTTAAGGGCACCGATTCCTGAAAGAATAAGTGGTTTAACTGGTGCTGTTAGATATTTAA
A L R A P I P E R **I S G L T G A V R Y L**

2761 CGCATATGGATAATCCTGAAAAGTATCAATACGATAATACAGAAATACAAGTGTTTGGTG
T H M D N P E K Y Q **Y D N** T E I Q V F G

2821 GATTTGACCTTGAAAGTTGTTTAGCGTTATCTACTGGTGATAAAAGACAAGCGTTAAAAG
G F D L E S C L A L S T G D K R Q A L K

2881 AAATGCTTGGTTTTATTTTCGGATAACAATATTATGCATTTAAAAGATTTTGCTGATTATT
E M L G F I S D N N I M H L K D F A D Y

2941 GTATGTCTGACCGAGCTCCTGCTGGTTGGTTTGAATTGCTAACAGAGAGGAATACTCTTT
C M S D R A P A G W F E L L T E R N T L

3001 TTATAAAAGAGTACATAAAATCGAATTGGCAAAAAGAAAACCAAGTTTATAAAGAG**TGAG**
F I K E Y I K S N W Q K E N Q V Y K E *

Figure 3

Figure 3

| | | Motif R-I | Motif R-II | Motif R-III | Motif R-IV | Motif R-V |
|---------|----|------------|--------------|----------------|-----------------------|-----------|
| pLFE1 | 29 | WWIVVYPESL | 52 AISPLHDKD | 68 KKPHYHIVFNY | 102 ISGLTGAVRYLTH-MDN | 121 YDN |
| pLB4 | 29 | WGIVVYPESL | 52 AVSPLHDKD | 68 KKSHYHLVLNY | 102 ISSLTGAVRYLTH-MDN | 123 YDN |
| pWV01 | 10 | FGFLLYPDSI | 35 AVSPLHDMD | 64 KKPHYHVI--Y | 102 LDYIKGSYEYLTHESKD | 127 YDK |
| pCI411 | 19 | WSWIVYPESA | 42 IESPLHDKD | 59 KKPHWHII-IS | 93 VASLQGSVQYLWHR-NN | 114 YDK |
| pLA106 | 13 | WTFVLYPESA | 38 VESPLHDKD | 54 KKPHWHIA-MF | 94 IENAKGMIRYFAH-MDN | 107 YQY |
| pE194 | 19 | WTFVLYPESA | 44 ALSPLHDKD | 60 KKEHYHILVMY | 95 AGSVKGLVRYMLH-MDD | 115 YQK |
| pHPK255 | 27 | FGFIIYPESA | 53 ALSPLHDKD | 69 KKPHFHAI-IV | 107 NERVKGAYEYFTH-SNP | 130 YDK |
| pPSC22 | 10 | FGFLLYPDSI | 35 AVSPLHDMD | 64 KKPHYHVI--Y | 102 LDYIKGSYEYLTHESKD | 127 YDP |
| pLS1 | 8 | FTFLLYPESI | 33 AISPLHDKD | 51 KKAHYHVL--Y | 89 VLNVENMYLYLTHESKD | 114 YDK |
| pJB01 | 8 | FTFLLYPESI | 33 AVSPLHDKD | 51 KKAHYHVI--Y | 90 STSMENMYLYLTHESKD | 115 YSK |

Figure 4

Figure 4

| | | strand I | helix A | turn | helix B | | |
|----------------|----|----------------------------|---------|------------------|---------|--|--|
| | | → | ● | — | ● | | |
| pLFE1-copG | 12 | VEKKKVTLSLPVESNDKLEKMAQKY | GMT | KSGLVTFLINQADDKG | 63 | | |
| pLB4-repA | 4 | VEKKKITLSIPVETNGKLEELAQKY | GMT | KSGLVNFLVNQVAEAG | 55 | | |
| pMV158-copG | 1 | MKKRLTITLSESVLENLEKMAREM | GLS | KSAMISVALENYKKGQ | 51 | | |
| pHD2-ORFB | 1 | MVRVNTRISKKLNDWLDEYSKES | GVP | KSTLVHLALENYVNQK | 50 | | |
| pCB101-repC | 1 | MRVNISIPDEVKQFFEDYSKKT | GVP | QSSLMALALSEYKDKI | 49 | | |
| pFX2-copX | 5 | ESKKRVMISLTKEQDKKLTDMAKQK | GFS | KSAVAALAIEEYA-RK | 57 | | |
| pHT926-unknown | 5 | QERERMQIRLSKTNMQRLQDMAGRY | GMS | ANSLVSYILGQWLDNN | 56 | | |
| pRN2-copG | 3 | STKPNVHIRLREEERKLLKEIAQKY | DIS | ESDVVKIALKKLAREL | 54 | | |
| pE194-cop6 | 8 | EKKVAVTLRLTTEENEILNRIKEY | NIS | KSDATGILIKKYAKEE | 59 | | |
| pA1-repA | 1 | MERVKVGITLTEDTLARLEEICKEM | GLS | KSQALSMLVNKEYLEK | 52 | | |
| pLA106-repA | 2 | TEKKRLTVSFESHKIANQLEELAKDQ | GLT | KSGLLTVLISKEIERK | 53 | | |

3